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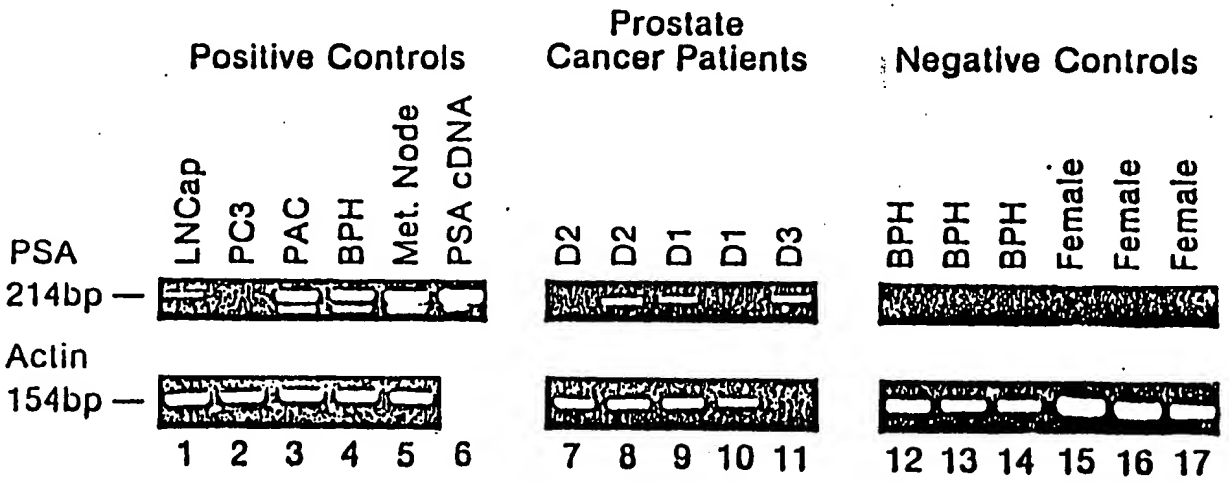
(54) Title: METHODS OF DETECTING MICROMETASTASIS OF PROSTATE CANCER



(57) Abstract

A method of diagnosing prostate metastasis is provided by the present invention whereby RNA from a patient's blood is isolated and amplified using a pair of primers which are complementary to regions of the prostate specific antigen gene. The presence or absence of amplified RNA is detected and the presence of amplified RNA is indicative micrometastasis of prostate cancer.

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Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

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METHODS OF DETECTING MICROMETASTASIS OF PROSTATE CANCER

FIELD OF THE INVENTION

This invention is directed to methods of detecting prostate cancer.

BACKGROUND OF THE INVENTION

Prostate cancer metastasis will claim the lives of over 30,000 Americans this year. Boring et al., *Cancer Statistics* **1991**, 19. The mode of dissemination however, remains very poorly understood. An almost dogmatic view of metastasis holds that prostate cancer cells first spread through the prostatic capsule then into the lymphatics, and eventually hematogenously travel to bone. Byar et al., *Cancer* **1972**, 30, 5; Winter, C.C., *Surg. Gynecol. Obstet.* **1957**, 105, 136; Hilaris et al., *Am. J. Roentgenol.* **1974**, 121, 832; McLaughlin et al., *J. Urol.* **1976**, 115, 89; Jacobs, S.C., *Urology* **1983**, 21, 337; Batson, O.V., *Ann. Surg.* **1940**, 112, 138; Saitoh et al., *Cancer* **1984**, 54, 3078-3084; Whitmore, W.F., Jr., *Cancer* **1973**, 32, 1104. However, this model has been based on histopathologic studies which have significant limitations, and in actuality the sequence of metastatic events remain unknown. Solid tumor animal experiments suggest that only 0.01% of circulating cancer cells eventually create a single metastatic deposit. Fidler et al., *Science* **1982**, 217, 998-1001; Liotta et al., *Cancer Res.* **1974**, 34, 997; Schirrmacher, B., *Adv. Cancer Res.* **1985**, 43, 1-32. Ostensibly, a single bone metastasis from human prostatic adenocarcinoma (PAC) could be generated by

10,000 circulating cancer cells (2 cells/1 ml blood). In the past, detection of such a low concentration of cells has been difficult or impossible. Recently, however, Wu et al. used keratin-19 (K-19) mRNA PCR to detect breast cancer micrometastasis in patient lymph nodes and bone marrow. Wu et al., *Lab. Inv.* **1990**, 62, 109A. Miyomura et al., also reported the detection of minimal residual acute lymphoblastic leukemia by PCR in patients harboring the Philadelphia chromosome. Miyomura et al., *Blood* **1992**, 79, 1366-1370.

A method of detecting the micrometastasis of prostate cancer would be greatly desirable.

SUMMARY OF THE INVENTION

In accordance with the present invention, methods of detecting prostate cancer micrometastasis in a patient are provided comprising the steps of obtaining a sample of RNA from a patient's blood and amplifying said RNA with polymerase chain reaction. The polymerase chain reaction is performed using a pair of primers which are complementary to separate regions of the prostate specific antigen gene. These primers may have the sequences GAGGTCCACACACTGAAGTT (SEQ ID NO: 1) and CCTCCTGAAGAATCGATTCCT (SEQ ID NO: 2). Thereafter, the presence or absence of amplified RNA is detected wherein the presence of amplified RNA indicates micrometastasis of prostate cancer.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows an agarose gel in which micrometastasis is indicated by the presence of a 214 base pair (bp) band.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with methods of the present invention, methods of detecting micrometastasis of prostate cancer in a patient is provided comprising the step of obtaining a sample of RNA from the patient's blood. Preferably the RNA is obtained from a blood sample such as a peripheral venous blood sample. A whole blood gradient may be performed to isolate nucleated cells and total RNA is extracted such as by the

RNazole B method (Tel-Test Inc., Friendswood, Texas) or by modification of methods known in the art such as described in Sambrook et al., *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989).

Thereafter, a polymerase chain reaction may be performed on the total extracted RNA. Preferably a reverse transcriptase PCR amplification procedure may be performed in order to quantify the amount of mRNA amplified. Polymerase chain reaction methodologies are well known in the art. Innis et al., *PCR Protocols*, Academic Press, Inc., San Diego CA, 1990. Polymerase chain reaction primers may be designed to be complementary to separate regions of the prostate specific antigen (PSA) gene. Henttu et al., *Biochem. Biophys. Res. Comm.* **1989**, 160, 903-910. By separate regions is meant that a first primer is complementary to a 3' region of the PSA gene and a second primer is complementary to a 5' region of the PSA gene. Preferably, the primers are complementary to distinct, separate regions and are not complementary to each other.

PSA is an important marker produced exclusively by prostatic epithelial cells and almost always expressed by prostate cancer. Stamey et al., *J. Urol.* **1989**, 141, 1076-1083. Thus, PSA2 (5-GAGGTCCACACACTGAAGTT, SEQ ID NO: 1) and PSA3 (5-CCTCCTGAAGAATCGATTCCT, SEQ ID NO: 2) oligonucleotide primers were designed to have high specificity to the PSA gene. A Gene Bank version-70 (Mountain View, CA) search confirmed the specificity of these primers to PSA and not to the human glandular kallikrein (HMGK) gene which has high homology to the PSA gene. Henttu et al, *Biochem. Biophys. Res. Comm.* **1989**, 160, 903-910. PSA2 and PSA3 bind sequences that span intron III of the PSA gene such that PCR amplification yields a 360 bp DNA and a 214 bp RNA product, thereby eliminating the possibility of false positives from DNA contamination. Oligonucleotide primers may be prepared by methods known in the art such as by standard phosphoramidite chemistry. (See Sambrook et al., *supra*). Following amplification, the presence or absence of mRNA amplification product may be detected. Preferably, the PCR product may be run on an agarose gel and

visualized using a stain such as ethidium bromide. (See Sambrook et al., *supra*).

The following examples are illustrative but are not meant to be limiting of the invention.

EXAMPLES

Example 1 Patient Specimens

Selection of cases was based on the following criteria. Prostate cancer patients were chosen for analysis if they had: (1) clinically and/or surgically staged DO-D2 disease (D0 = elevated tumor markers with no demonstrable metastasis, D1 = pelvic lymph node involvement, D2 = disseminated disease usually to bone) without having received prior hormonal therapy and who had an elevated serum PSA, or (2) stage D3 disease (D2 disease that is refractory hormonal therapy) with an elevated PSA Negative control patients consisting of female volunteers, and patients with benign prostatic hypertrophy (BPH) proven by biopsy or men who were on a BPH study protocol. Patients who had surgical manipulation of the prostate during the previous year were excluded from the study. Positive controls included a lymph node from a patient with known metastatic PAC tissue from pathologically proven BPH and cDNA PSA plasmid. Henttu et al, *Biochem. Biophys. Res. Comm.* **1989**, 160, 903-910. The protocol was IRB approved and written consent was obtained. LNCAP and PC3 human cell lines were obtained from The American Type Culture Collection, (Rockville, MD).

Example 2 Blood Preparation for RNA Extraction

Approximately six ml of venous blood were obtained with a standard venipuncture technique using heparinized tubes. Whole blood was mixed with an equal volume of phosphate buffered saline (PBS) which was then layered over eight ml of Ficoll (Pharmacia Uppsala, Sweden) in a 15 ml polystyrene tube. The gradient was centrifuged at 200 g for 30 minutes at 5°C. The lymphocyte and granulocyte layer (approximately 5 ml) was carefully aspirated and re-diluted up to 50 ml with PBS in a 50 ml tube which was then centrifuged at 1800 g for 20 minutes a

5°C. Supernatant was discarded, and the pellet containing nucleated cells was used for RNA extraction using the RNazole B method, as described by the company (Tel-Test Inc., Friendswood, Texas).

Example 3 Oligonucleotide primers and probes

PSA2 (5-GAGGTCCACACACTGAAGTT, SEQ ID NO: 1) and PSA3 (5-CCTCCTGAAGAATCGATTCCT, SEQ ID NO: 2) oligonucleotide primers were custom designed with high specificity to the PSA gene; a Gene Bank version-70 (Mountain View, CA) search confirmed the specificity of these primers to PSA and not to the human glandular kallikrein (HMGK) gene which is 75-85% homology to the PSA gene. Henttu et al, *Biochem. Biophys. Res. Comm.* 1989, 160, 903-910. All primers were synthesized and gel purified by the City of Hope DNA Synthesis Laboratory (Duarte, California). PSA2 and PSA3 bind sequences that span intron III such that PCR amplification yielded a 360 bp DNA and a 214 bp RNA product. Previously published actin PCR primer sequences were used to rule out degraded RNA, and amplification with actin oligonucleotide primers A1 and A2 yielded a 154 bp RNA and a 250 bp DNA product. Ben-Ezra et al., *J. Histochem Cytochem.* 1991, 39, 351-354.

Example 4 Polymerase Chain Reaction

The reverse transcriptase reaction and PCR amplification were performed sequentially without interruption in a Perkin Elmer 9600 PCR machine (Emeryville, CA). 400 ng of total RNA in 20 µl DEPC (Diethyl-pyrocarbonate) treated water were placed in a 65°C water bath for five minutes then quickly chilled on ice immediately prior to the addition of PCR reagents. The 50 µl total PCR volume consisted of 2.5 units Taq polymerase (Perkin Elmer, Emeryville, CA), 2 units AMV reverse transcriptase (Boehringer Mannheim, Indianapolis, IN), 200 µM each of dCTP, dATP, dGTP, and dTTP (Perkin Elmer, Emeryville, CA), 18 pM each primer, 10 mM Tris-HCL, 50 mM KCl, 2 mM $MgCl_2$ (Perkin Elmer, Emeryville, CA). PCR conditions were as follows: cycle 1 was 42°C for 15 minutes, then 97°C for 15

seconds (one cycle); cycle 2 was 95°C for one minute, then 60°C for one minute and 72°C for 30 seconds (15 cycles); cycle 3 was 95°C for one minute, then 60°C for one minute, and 72 degrees for one minute (10 cycles); cycle 4 was 95°C for one minute, then 60 for one minute and 72°C for two minutes (8 cycles); cycle 5 was 72°C for 15 minutes (one cycle); and the final cycle was a 4°C hold until sample was taken out of the machine. The 50 μl PCR products were concentrated down to 10 μl with vacuum centrifugation and the entire sample was then run on a thin three percent Tris-borate-EDTA (TBE) agarose gel containing ethidium bromide. All specimens were analyzed at least twice to confirm a positive or negative outcome.

The potential risk of false positives from cross contamination was avoided by performing RT PCR in a single tube without interruption and using filtered pipet tips. Sensitivity was enhanced by using high amounts of Taq polymerase, progressively increasing extension times, and analyzing the entire 50 μl PCR product on thin ethidium bromide agarose gels. These measures ensured a high fidelity assay while maintaining technical simplicity.

Prostate human tissue specimens, tissue culture cell lines and a PSA cDNA plasmid, cloned and described by Henttu and Vihko; Henttu et al., *Biochem. Biophys. Res. Comm.* **1989**, 160, 903-910, were used as positive controls, and they demonstrated the 214 bp bands as shown in fig.1 top panel. A pelvic lymph node with metastatic PAC, a primary prostate cancer, and a BPH specimen all produced strong PSA PCR signals. The LNCAP and PC-3 human prostate cell lines produced weaker signals.

EXAMPLE 5 Sequencing

Specificity of these primers to the PSA gene was confirmed with DNA sequence analysis of the amplified 214 bp fragment (Figure 1 bottom panel) which in this segment had very little homology to the HMGK gene. The 214 bp product was purified with a Qiagen PCR Product Purification kit (Qiagen, Chatsworth, CA) as described by the manufacturer. One microgram

of the PCR product underwent a PCR sequencing reaction by using the Taq DyeDeoxy Terminator Cycle sequencing kit in a Perkin-Elmer 9600 PCR Machine, as described by Applied Biosystems (Applied Biosystems, Foster, CA). The sequenced product was purified using centri-sep columns (Princeton Separations, Adelphia, New Jersey) as described by the company. This product was then analyzed with a ABI Model 373A DNA sequencing system (Applied Biosystems, Foster, CA) integrated with a Macintosh IIci computer.

Example 6 Detection of Circulating Hematogenous Micrometastasis

Twelve prostate cancer patients and 17 control patients underwent RT PCR analysis on PSA and actin RNA extracted from blood, as described in Examples 1 through 4 (Table 1). All cases demonstrated satisfactory RNA quality by actin PCR (Figure 1, bottom row). Of the 12 human prostatic adenocarcinoma (PAC) patients with metastatic disease, four cases (33%) had positive PSA signals indicating the presence of prostatic epithelial cells in the peripheral venous blood. These four cases consisted of two stage D1 patients, one stage D2 patient, and one stage D3 patient (N=1) (Figure 1, top row). The 17 negative controls, which consisted of eight volunteer women and nine men with BPH, all had undetectable PSA mRNA by RT PCR. These data indicate that RT PCR of the PSA RNA gene can be used to specifically detect circulating hematogenous micrometastasis in patients with stage D1-D3 pathology. These findings are in agreement with studies by Hamby et al. who detected circulating PSA positive cells in patients with metastatic prostate cancer by flow cytology and immunohistology. Hamby et al., *Br. J. Urol.* 1992, 69, 392-396.

Micrometastasis was not detected in eight of twelve prostate cancer patients consisting of two stage D3 patients, two stage D1 patients, and four stage D0 patients. In order to enhance the detection of micrometastasis, analysis may focus on buffy coat cells. Results indicate that the prostate cancer cells may be more concentrated in the "buffy coat". The PSA

signal was stronger in the RNA extracted from cells obtained only from the "buffy coat" (Figure 1, lane 8) compared to those isolated from the entire Ficoll layer (Figure 1, lane 7) in the same prostate cancer patient. These findings are in agreement with those of Harty et al. who found that prostatic epithelial cells migrate into the "buffy coat". Harty et al., *J. Surg. Res.* **1979**, 26, 411-416.

SEQUENCE LISTING

(1) GENERAL INFORMATION:
(i) APPLICANT: Croce et al.

(ii) TITLE OF INVENTION: Methods of Detecting Micrometastasis Of Prostate Cancer

(iii) NUMBER OF SEQUENCES: 2
(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Woodcock Washburn Kurtz Mackiewicz & Norris
(B) STREET: One Liberty Place - 46th Floor
(C) CITY: Philadelphia
(D) STATE: PA
(E) COUNTRY: USA
(F) ZIP: 19103
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(B) COMPUTER: IBM PS/2
(C) OPERATING SYSTEM: PC-DOS
(D) SOFTWARE: WORDPERFECT 5.1
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(B) FILING DATE:
(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Lori Y. Beardell
(B) REGISTRATION NUMBER: 34,293
(C) REFERENCE/DOCKET NUMBER: TJU-0722
(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (215) 568-3100
(B) TELEFAX: (215) 568-3439

(2) INFORMATION FOR SEQ ID NO: 1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20
(B) TYPE: Nucleic
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(iv) ANTI-SENSE: No
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:
GAGGTCCACA CACTGAAGTT 20

(2) INFORMATION FOR SEQ ID NO: 2:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21
(B) TYPE: Nucleic
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(iv) ANTI-SENSE: No
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:
CCTCCTGAAG AATCGATTCC T 21

What is claimed is:

1. A method of detecting prostate cancer micrometastasis in a patient comprising the steps of:

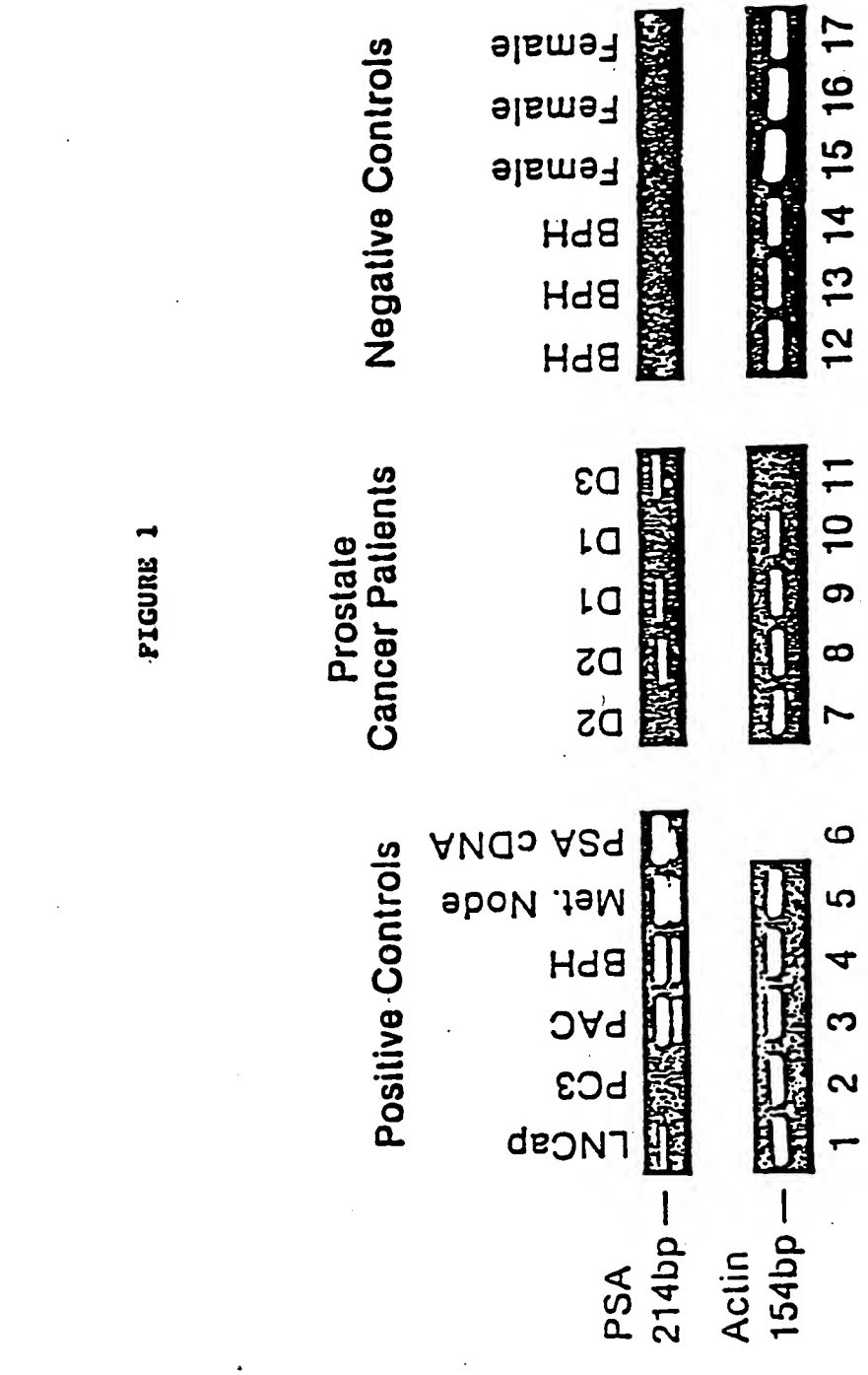
obtaining a sample of RNA from a patient's blood;

amplifying said RNA with polymerase chain reaction using a pair of primers which are complementary to separate regions of the prostate specific antigen gene; and

detecting the presence or absence of amplified RNA wherein the presence of amplified RNA indicates micrometastasis of prostate cancer.

2. The method of claim 1 wherein said primers have the sequences GAGGTCCACACACTGAAGTT (SEQ ID NO: 1) and CCTCCTGAAGAATCGATTCCT (SEQ ID NO: 2).

3. The method of claim 1 wherein said RNA is obtained from cells from the buffy coat of the Ficoll layer of the prepared blood sample.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D1
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Negative Controls
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1 2 3 4 5 6
7 8 9 10 11
12 13 14 15 16 17

FIGURE 1

INTERNATIONAL SEARCH REPORT

I national application No.
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A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12Q 1/68
US CL :435/6, 91.1; 935/77, 78

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.1; 935/77, 78

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, GENEBANK, EMBL, GENESEQ, CA, BIOSIS, MEDLINE, EMBASS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	Cancer Research, Vol. 52, Number 21, 01 November 1992, MORENO ET AL., "Detection of Hematogenous Micrometastasis in Patients with Prostate Cancer", pages 6110-6112, see the entire document.	1-3
Y	British Journal of Urology, Vol. 69, issued 1992, HAMDY ET AL., "Circulating Prostate Specific Antigen-positive Cells Correlate with Metastatic Prostate Cancer", pages 392-396, see the Abstract and Figure 1.	1-3

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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PCT/US93/10331

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nucleic Acids Research, Vol. 17, No. 5, issued 1989, GOBLET ET AL., "One-step Amplification of Transripts in Total RNA Using the Polymerase Chain Reaction", page 2144, see Panel A.	1-3
Y	Journal of Surgical Research, Vol. 26, issued 1979, HARTY ET AL., "In Vitro Technique for Isolating Prostate Cells from Blood", pages 411-416, see Materials and Methods.	1-3
Y	Biochemical and Biophysical Research Communications, Vol. 159, No. 1, issued 28 February 1989, RIEGMAN ET AL., "Characterization of The Prostate-Specific Antigen Gene: A Novel Human Kallikrein-Like Gene", pages 95-102, see Figure 2.	1-3
Y	Current Opinion in Immunology, Vol.4, issued 1992, O'GARRA ET AL., "Polymerase Chain Reaction for Detection of Cytokine Gene Expression", pages 211-215, see Figures 1 and 2.	1-3

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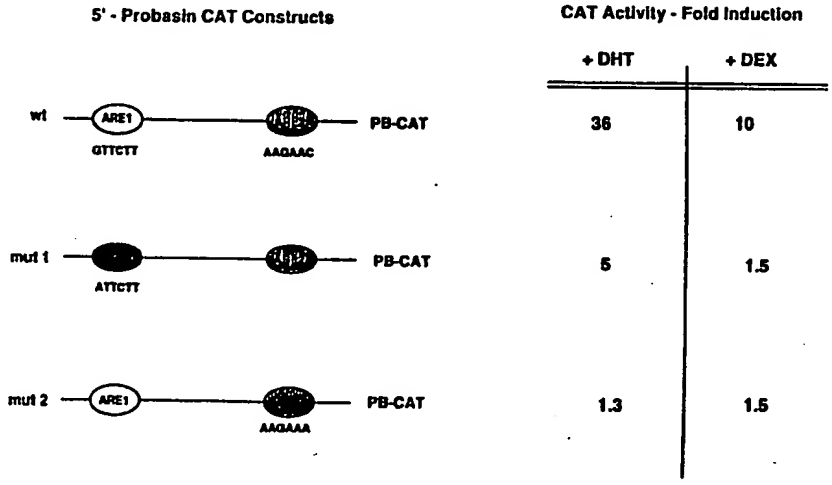
(54) Title: ANDROGEN REGULATION WITH DNA SEQUENCES OF RAT PROBASIN GENE



(57) Abstract

A DNA sequence (-426 to +28 base pairs) cloned from the probasin (PB) gene promoter region confers androgen regulation in cell culture and prostate specific expression in transgenic non-human eukaryotic animals. Various PB promoter fragments impart preferential regulation by androgens compared to other steroid hormones on fused transgenes. Alteration of the DNA sequences and/or combinations permits production of an (1) assay for androgenic or anti-androgenic materials, (2) transgenic non-human eukaryotic animals models for prostatic disease, (3) cell culture models for prostatic disease, and (4) treatment of human benign prostatic hyperplasia and human prostate cancer by gene therapy. This invention permits assays on agonist and antagonist of the androgen receptor or pathways that result in androgen action, testing materials for carcinogenicity of the prostate, testing drugs and gene therapy, or protection potential of materials on prostatic cells against prostatic disease.

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ANDROGEN REGULATION WITH DNA SEQUENCES OF RAT PROBASIN GENE

FIELD OF THE INVENTION

The present invention is concerned with a novel DNA molecule and fragments thereof, which permits production of an (1) assay for androgenic or anti-androgenic materials, (2) transgenic non-human eukaryotic animals models for prostatic disease, (3) cell culture models for prostatic disease, and (4) treatment of human benign prostatic hyperplasia and human prostate cancer by gene therapy. This invention permits assays on agonist and antagonist of the androgen receptor or pathways that result in androgen action, testing materials for carcinogenicity on the prostate, testing drugs and gene therapy, or protection potential of materials on prostatic cells against prostatic disease.

BACKGROUND OF THE INVENTION

Androgen Activity: A clinical need to assay the function of the androgen receptor (AR) occurs when defects appear in the pathway of androgen action. For example, mutations in the AR affect the bioactivity of the receptor in Androgen Insensitivity Syndrome, AIS, (Kazemi-Esfarjani et al., 1993; Brinkmann et al., 1991; Brinkmann et al., 1992a; Brinkmann et al., 1992b; De Bellis et al., 1992; French et al., 1990; Imperato-McGinley et al., 1990; Lubahn et al., 1989; Quigley et al., 1992; Ris-Stalpers et al., 1990; Ris-Stalpers et al., 1991; Simental et al., 1992) or testicular feminized animals (Yarbrough et al., 1990; He et al., 1991), Kennedy Syndrome (La Spada et al., 1991), prostate cancer (Newmark et al., 1992; Brinkmann et al., 1991; Veldscholte et al., 1992b; Veldscholte et al., 1992a; Veldscholte et al., 1990) and breast cancer (Wooster et al., 1992). Besides mutations directly in the receptor, defects can occur in the non-androgenic mechanism for steroid receptor activation as has been

reported for steroid receptors (Power et al., 1991; Shemshedini et al., 1992; Kuiper et al., 1993). An assay that would measure the extent of these defects would also provide a tool to test new materials that may activate the defective receptor and form the basis of a therapy.

Androgen receptors are members of a nuclear receptor superfamily which are believed to function primarily as transcription factors that regulate gene activity through binding specific DNA sequences to hormone responsive elements (HRE) and associated factors (Allan et al., 1991; Smith et al., 1993; Evans, 1988; Beato, 1989). In general, these HREs can be grouped into two categories of inverted repeat consensus sequences: the TGACC motif that mediates estrogen, retinoic acid, and thyroid hormone responses (Klein-Hitpass et al., 1986; Umesono et al., 1988); and the TGTTCT sequence that confers regulation by glucocorticoids, progestins and androgens (Scheidereit et al., 1986; Shrahle et al., 1987; Ham et al., 1988). The inclusion of the androgen receptor responsive element (ARE) in this latter group is based largely on observed binding of androgen receptors to the glucocorticoid responsive element (GRE) of mouse mammary tumor virus (MMTV) DNA (Ham et al., 1988; Roche et al., 1992; Darbre et al., 1986; Cato et al., 1987) and the tyrosine aminotransferase (TAT) gene (Denison et al.,1989).

Androgen regulation of the C3(1) gene which encodes a polypeptide component of prostatic steroid-binding protein has been investigated (Heyns et al., 1978; Hurst et al., 1983; Parker et al., 1988; Parker et al., 1980). Although sequences within both the promoter region and first intron of the C3(1) gene have high affinity binding for androgen receptors (Perry et al., 1985; Claessens et al., 1993; De Vos et al., 1991; Rushmere et al., 1990), attempts to use these sequences to confer androgen regulation on a homologous or heterologous promoter-reporter system have met with limited success

(Parker et al., 1985; Parker et al., 1988); with only a weak androgen induction seen with these genomic fragments (Claessens et al., 1993; Tan et al., 1992; De Vos et al., 1991; Rushmere et al., 1990; Claessens et al., 1990b; Claessens et al., 1990a; Claessens et al., 1989b; Claessens et al., 1989a). Recently, DNase I footprinting experiments have shown that the DNA-binding domain of the androgen receptor binds to a glucocorticoid responsive element (GRE) present in this intronic fragment (De Vos et al., 1991; Claessens et al., 1993). The occurrence of a complete GRE in this gene is consistent with the observed effects of glucocorticoids on the expression of the C1 component of prostatic binding protein (Rennie et al., 1989). The human prostate specific antigen (PSA) gene is androgen regulated in human prostate tumors and in cell culture (Riegman et al., 1991; Montgomery et al., 1992; Young et al., 1992; Murphy et al., 1992; Armbruster, 1993). Construction of the PSA DNA promoter reveal a GRE-like sequence that responds to androgens (Riegman et al., 1991). The Slp gene demonstrates specific androgen regulation via GRE-like sequences (Adler et al., 1992; Adler et al., 1991). Other androgen regulated genes from the prostate have been cloned, such as the SVS II (Dodd et al., 1983; Dodd et al., 1986; Harris et al., 1990), 20 kDa protein (Ho et al., 1989), and DP1 (Ho et al., 1992), but the androgen regulatory sequences have not been identified.

Transgenic animals: The introduction of a gene into the germline at the one cell or early embryonic stage produces a transgenic animal which will contain and pass on the gene to its offspring. Tissue specific expression of a gene can be restricted by tissue specific elements with the DNA. Success with prostate specific expression of transgenes has been limited and often not restricted to the prostate. For example, the complete rat C3(1) gene including 4.3 kb of 5'-flanking sequence and 2.2 kb

of 3'-flanking sequence will give prostate specific expression in transgenic mice (Allison et al., 1989), but using only the 6 kb of 5'-flanking C3(1) resulted in transgenic lines that targeted to the prostate, seminal vesicles, and testis (Buttyan et al., 1993). MMTV coupled to int-2 produced a transgenic mouse line that developed prostatic epithelial cell hyperplasia that was androgen regulated but the males are sterile (Muller et al., 1990; Leder, 1990; Tutrone et al., 1993). Various males in different lines, in addition to expressing the transgene in the prostate, also expressed the int-2 gene in the seminal vesicles, vas deferens, salivary gland while the females expressed the gene in the mammary gland and developed mammary hyperplasia (Leder, 1990). However, targeting with MMTV can lead to expression in the testis resulting in sterility (Lucchini et al., 1992). Using the gp91-phox gene promoter (a gene not normally expressed in the prostate) linked to the early region of SV-40 virus, lesions in the prostate defined as neuroblastomas were created (Skalnik et al., 1991) .

Any gene targeted to the prostate in transgenic animals may alter prostatic growth and function. Oncogenes and tumor suppressor genes (Fleming et al., 1986; Matusik et al., 1987; Dodd et al., 1990; Hockenbery, 1992; Buttyan et al., 1993; Carter et al., 1990a; Carter et al., 1990b; Tutrone et al., 1993; Bookstein et al., 1993; Thompson et al., 1993; Peehl, 1993; Dodd et al., 1993; McNicol et al., 1991; McNicol et al., 1990a; McNicol et al., 1990b) as well as growth factors (Morris et al., 1990; Ichikawa et al., 1992; Isaacs et al., 1991a; Isaacs et al., 1991b; Carter et al.,1990a; Pienta et al., 1991; Morton et al., 1990) implicated in the development of prostatic hyperplasia or cancer are likely starting points. In addition, genes such as the large T antigen, which successfully induce cancer in endocrine glands when targeted in transgenic

animals, are suitable candidates (Anonymous, 1991; Stefaneanu et al., 1992; Hanahan, 1986; Rindi et al., 1991; Hamaguchi et al., 1990).

Transgenic animals that express the transgene in a tissue or non-tissue specific can result in new models. For example, non-tissue specific expression can result in diseased states in a number of tissues while tissue specific expression of targeted genes can lead to disease states in targeted organs as follows: cancer models (Burck et al., 1988; Yamamura, 1989; Folkman et al., 1989; Reynolds et al., 1988; Anonymous, 1992; Bautch, 1989; Hanahan, 1986; Lucchini et al., 1992; Anonymous, 1988); mammary adenocarcinoma (Muller et al., 1988; Muller, 1991; Pawson, 1987; Callahan et al., 1989; Muller, 1991; Strange et al., 1990); hyperplasia and dysplasia (Mayo et al., 1988; Borrelli et al., 1992; Eva et al., 1991; Lin et al., 1992; Matsui et al., 1990); neuroblastomas (Dalemans et al., 1990); liver cancer (Butel et al., 1990; Dubois et al., 1991; Sandgren et al., 1993; Sandgren et al., 1989); gonadal tumors (Schechter et al., 1992; Matzuk et al., 1992); thymic mesenchymal tumors (Sinkovics, 1991); and leukaemia (Knight et al., 1988; Adams et al., 1985). Further, targeted genes may function to accelerate tumor formation by conferring susceptibility to transformation by factors or carcinogens (Langdon et al., 1989; Breuer et al., 1989; Mougneau et al., 1989). Promoters, such as metallothionein (MT), often lead to general expression in many organs (Dyer et al., 1989; Iwamoto et al., 1991) while the MMTV promoter limits expression to endocrine target tissues due to its HRE (Ham et al., 1988; Roche et al., 1992; Darbre et al., 1986; Cato et al., 1987). Even using a general promoter can lead to specific effects if the factor expressed targets a specific tissue, i.e. MT-growth hormone releasing factor (Mayo et al., 1988) or

ectopic nerve growth factor (Borrelli et al., 1992) lead to pituitary hyperplasia in transgenic mice.

Gene therapy: The treatment of human disease or disease in non-human eukaryotic animals by gene therapy started with the goal to correct single-gene inherited defects. Advances have expanded that goal to include the treatment of acquired diseases, such as cancers (Davies, 1993; Anderson, 1992; Mulligan, 1993; Culotta, 1993; Felgner, 1993; Tolstoshev et al., 1993). Approved clinical trials are presenting encouraging results. The practical problem has been the development of efficient and specific approaches that will transfer and express a gene within the correct cell type. The approaches can be classed as viral and nonviral methods to transfer genes. Some of the therapeutic approaches transfer the gene(s) to patient cells which have been cultured and then returned to the same individual (Fenjves et al., 1989). Others attempt direct transfer of the gene to the human tissue. For example, a DNA complex with liposomes can be delivered to the airway and correct the cystic fibrosis defect in transgenic mice (Hyde et al., 1993). Direct gene transfer by DNA: cationic liposomes into adult mice demonstrates efficient transfer and expression occurs in most organs (Zhu et al., 1993). If the gene is stably integrated, then the defect may be corrected while, if the gene was transiently expressed, then a relief in the disease would likely be transient. However, in cases, such as cancer, where the goal is to kill the cancerous cell, transient expression would be sufficient if the expressed gene is toxic (Short et al., 1990; Culver et al., 1992). Approaches may include expressing tumor suppressor genes (Friedmann, 1992) or genes to inhibit expressed oncogenes (Mukhopadhyay et al., 1991).

SUMMARY OF THE INVENTION

In one aspect, the present invention provides an isolated DNA molecule comprising a 5'-flanking region of

the rat probasin gene and containing at least one androgen responsive element, preferably two such elements. The DNA molecule preferably has the sequence shown in Figure 1 (SEQ ID NO: 1) or one which hybridizes thereto under stringent conditions (SEQ ID NO: 2 shows the derived amino acids for the amino acid coding portion of the DNA molecule).

In another aspect of the invention, there is provided an isolated DNA molecule comprising an androgen responsive element of the rat probasin gene or a mutation thereof retaining androgen activity, preferably, the DNA sequence comprises nucleotides -241 to -223 and/or nucleotides -140 to -117, as seen in Figure 1, or a mutation thereof retaining androgen activity. In one embodiment, nucleotides -130 to -127 are replaced by nucleotides TACT (SEQ ID NO: 3) or GTCT (SEQ ID NO: 4).

The present invention includes not only the isolated and purified PB nucleotide sequences but also includes (1) an assay for androgenic or anti-androgenic materials, (2) transgenic non-human eukaryotic animals models for prostatic disease, (3) cell culture models for prostatic disease, (4) treatment of human benign prostatic hyperplasia and human prostate cancer employing such DNA sequences. The present invention permits assays on agonist and antagonist of the androgen receptor or pathways that result in androgen action, testing materials for carcinogenicity on the prostate, testing drugs and gene therapy, and protection potential of materials on prostatic cells against prostatic disease.

First, the Figures and Tables will be described. Unless otherwise stated, all bioassays of PB constructs are performed in PC-3 cells with the cotransfection of the appropriate steroid receptor expression vector (Rennie et al., 1993; Kazemi-Esfarjani et al., 1993).

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 shows the PB rat genomic sequence from -426 to +28 bp (SEQ ID NO: 1). The start of transcription is shown as v with the number starting immediately after as +1. All negative numbering is relative to the start site of transcription. Sequences beyond +28 define additional leader sequences and amino acid sequence of the first exon for PB. By sequence homology, the GRE-like sequence (ARE-1), CAAT box (SEQ ID NO: 5), and TATAA box (SEQ ID NO: 6) are underlined.

Figure 2 shows the deletion mapping of PB 5'-flanking sequences (-426, -346, -307, -286, -244, -235, -157 bp) in PB-CAT, co-transfected in PC-3 cells with rAR or rGR expression vectors plus DHT (solid circles) or DEX (solid triangles), respectively. Activity of CAT with the rAR (open circles) or rGR (open triangles) expression vector without hormone addition served as the baseline (lower panel).

Figure 3 shows the CAT activity of wild type (wt) AREs (ovals) in -244 PB-CAT compared to single base mutation (hatched ovals) in either ARE-1 (mut 1) or ARE-2 (mut 2). Activities are shown as fold induction from baseline for DHT and DEX with their respective steroid receptor in PC-3 cells.

Figure 4 shows the bioassay of wild type human AR compared to when valine-865 is substituted by methionine (V865M) or leucine (V865L) which results in complete or partial AIS, respectively.

BRIEF DESCRIPTION OF TABLES

Table I shows the hormonal induction of PB-CAT in HeLa and PC-3 cells with DHT, DEX, and progestin.

Table II shows that the specific/tight AR binding is dependent upon both ARE sites as well as adjacent DNA sequences.

Table III shows that the bioactivity and specificity for AR is dependent upon both AREs as well as adjacent DNA sequences.

Table IV shows the bioactivity of -286 PB-ARE2*CAT compared for wild type -286 PB-CAT for wild type AR and when valine-865 is substituted by methionine or leucine in AIS.

Table V shows the bioassay effect on androgen induction when repeats -244 to -96 adjacent to TK-Luc.

Table VI shows the sensitivity increase to very low levels of androgen compared to the glucocorticoid DEX when the repeat n=3.

Table VII shows the sensitivity increase of repeat n=3 to detect differences in the wild type human AR from AIS samples.

Table VIII shows the high PB-CAT activity in the prostatic lobes of transgenic line #4248.

Table IX shows PB-CAT transgenic line #4248 expression in the mouse compared the endogenous rat PB gene. Expression in lateral lobe is taken as 100% and all other expression is compared relative to this value.

Table X shows the PB-CAT transgenic line #4248 expression after castration and androgen or glucocorticoid replacement.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

1) Assay for Androgenic or Anti-Androgenic Materials.

The inventor has isolated and sequenced (Figure 1) the 5'-flanking DNA of the rat probasin (PB) gene which is expressed specifically in the prostatic lobes and detectable in the seminal vesicles (Matusik et al., 1986). The probasin gene codes for a secreted and nuclear protein (Spence et al., 1989), which is androgen regulated *in vivo* and *in vitro* (Dodd et al., 1983; Rennie et al., 1993).

Isolation of Genomic Probasin Clone

To isolate the 5'-flanking DNA of the PB gene, pM-40 (Dodd et al., 1983), a cDNA clone which contains the complete coding region for PB (Spence et al., 1989), was used to screen a rat genomic library. Four positive clones were initially isolated and found to be identical after restriction and hybridization analysis. After subcloning into pUC119, the genomic clones were bidirectionally sequenced. The sequence of the 5'-flanking DNA of PB between -426 to +28 base pair (bp) is shown in Figure 1. The 5' boundary of exon 1 was defined by both primer extension and S1 nuclease mapping (Spence et al., 1989). A major transcription start site (position 0) is followed four bp downstream by a minor start site. The canonical CAAT and TATAA boxes are underlined in Figure 1 and located at −48 and −27 respectively. Four similar CAAT boxes are found at −101, −95, −82, and −75.

DNase I Footprinting Analysis

The DNase I footprinting was performed essentially as described (Von der Ahe et al., 1993; Rennie et al., 1993). Proteins (10 ng to 18 μg) were incubated with 20,000 cpm of the labelled DNA in 100 μl of DNA binding buffer for 30 min at 20°C. After adjusting the samples to 4 mM $MgCl_2$ and 2.5 $CaCl_2$ mM, 5 ng of DNase I was added and allowed to digest the DNA for 2 minutes at 20°C. The reaction was stopped by adding 100 μl of stop buffer (0.25% SDS, 0.3 M EGTA, and 500 μg/ml of proteinase K) and incubated for 1 hour at 37°C. The samples were extracted with phenol-chloroform; precipitated with 0.2 M sodium acetate, 85 μg/ml carrier tRNA, and 2 volumes of ethanol; redissolved in 3 μl of formamide dye solution. After heating at 70°C for 10 minutes and rapid cooling on ice, the samples were loaded, together with an A+G Maxam-Gilbert sequencing reaction to obtain a purine ladder, on 7% polyacrylamide/urea gels (acrylamide:bis, 30:1) and

run (Rennie et al., 1993). The gels were dried and set up for autoradiography.

The androgen response elements (ARE) are located at -241 to -223 (ARE-1) and -140 to -117 (ARE-2). The sequence of ARE-1 and ARE-2 in the 5'-flanking PB DNA were determined using DNase I footprinting assays with peptides containing the DNA-binding domain of the androgen receptor and later bioassay of the functional domains. The DNA- and steroid-binding domains of the rat androgen receptor (GST-AR1) and the DNA-binding domain and hinge region alone (GST-AR2) were expressed in *E. coli* as fusion proteins with glutathione-S-transferase and purified using glutathione affinity chromatography (Rennie et al., 1993). Both GST-AR1 and GST-AR2 gave qualitatively similar DNase I footprinting patterns revealing two binding sites: one between positions -236 and -223 (by 5'-deletion mapping, the DNA bases extending to -244 were found to be necessary for bioactivity); and the other between -140 and -117 (ARE-2). Both androgen receptor binding sites are similar to glucocorticoid responsive elements (GRE) with a conserved 5'GTTCT, synthetic ARE oligomers were much more efficient competitors in band shift for binding to probasin DNA than those corresponding to the glucocorticoid (GRE; weak competitor) or estrogen (ERE; inactive competitor) responsive elements (Rennie et al., 1993). Further, by homology to the GRE, the PB ARE-1 was defined as -241 to -223 (ATAGCATCTTGTTCTTAGT - SEQ ID NO: 7) whereas ARE-2 encompasses GRE like sequences in -140 to -117 (GTAAAGTACTCCAAGAACCTATTT - SEQ ID NO: 8).

Construction of Chimeric CAT Gene

The plasmid, pPH 1.4, containing the PB 5'-flanking sequence beginning at the *Hind III* site (-426 bp), exon 1, and part of intron A ending at the *Pst I* site (Rennie et al., 1993), was digested with *Sac I* to remove the coding region of exon 1 and intron A. The *Sac I* site was

blunt-ended with Klenow DNA polymerase and ligated to a *Bam HI* linker. After transformation into *E. coli* and screening for appropriate clones, the plasmid pBH 500 in the vector pU119 was obtained. The PB-CAT chimeric gene was constructed by inserting the bacterial chloramphenicol acetyl transferase (CAT) gene, prepared from a *Bam HI/Bgl II* digestion of p-109TK (Cattini et al., 1986), into the *Bam HI* site of pBH500, creating the -426 to +28 PB sequences adjacent to CAT. This construct also contains the SV40 sequences which provide a 3' intron and polyadenylation and cleavage signals. After transformation into *E. coli* and screening, suitable clones containing the plasmid designated as -426 PB-CAT were isolated. Deletions of -426 PB-CAT were prepared from a *Hind III* digest followed by a time course treatment with Bal 31 exonuclease (15, 30, 45, 60, and 75 seconds). The *Hind III* site was reconstituted by ligation to *Hind III* linkers. After transformation into *E. coli*, clones containing deletion mutants were screened and their plasmid DNAs digested with *Hind III* and *Eco RI* to determine fragment sizes by electrophoresis in 1.5% agarose gels. A range of deletion mutants was picked as a result of this enzyme digestion size screening. The original clones were then double digested with *Hind III* and *Bam HI*, and the insert isolated after electrophoresis in low melting point agarose gels. Subsequently, the *Hind III/Bam HI* fragments were subcloned into the *Hind III* and Bam HI sites of an undeleted pUC119 plasmid vector. The amount of PB bp in each construct (Figure 2) is labelled with a negative and/or positive number from the sequence depicted in Figure 1. The nucleotide sequences of the construct were confirmed by dideoxy sequencing.

Further PB-CAT chimeric constructs were made by replacing the endogenous PB promoter with the TK gene promoter. The PB fragments were obtained by the

appropriate restriction enzyme digest. One construct was made by PCR amplifying the -244 to -96 region using one primer containing a *Hind* III site at the 5' end and the other primer containing an *Xba* I site at the 3' end (Rennie et al., 1993). This polymerase chain reaction (PCR) amplified fragment was forced orientation subcloned into *Hind* III/*Xba* I TKCAT, generating the chimeric plasmid -243/-96PB-TKCAT. Mutations were made using Muta-Gene Phagmid *in vitro* mutagenesis kit (Bio-Rad, Mississauga, Ontario, Canada). The nucleotide sequences of all constructs were confirmed by dideoxy sequencing.

Cell Culture and Transfections

HeLa cells were plated at an initial density of $2x10^6$/100 mm dish in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS) or 5% FCS and 5% calf serum. The PC-3 cells were plated at an initial density of $8X10^5$/100 mm dish in Minimal Essential Media (MEM) supplemented with 10% FCS. Transient transfection of the HeLa cells with plasmid DNA was performed using a calcium phosphate/DNA precipitation method (Cattini et al., 1986). The cells were treated with 20% glycerol/DMEM for 2 minutes at 6 hours post-transfection and were subsequently grown in DMEM plus 1% charcoal stripped FCS (STR-FCS) with or without dihydrotestosterone (DHT), or dexamethasone (DEX) for 24 or 40 hours before cells were harvested. Transient transfection of PC-3 were performed as outlined for HeLa cells except that the PC-3 cells were grown in MEM plus 5% STR-FCS.

CAT Assays

Cells which had undergone transient transfection were harvested in phosphate buffered saline containing 1 mM EDTA. After centrifugation at 2000 xg for 4 min at room temperature, cells were lysed in 0.1 M Tris-HCl/0.1% Triton X-100, pH 7.8 for 15 min on ice. Insoluble material was removed by centrifugation at 14,500 xg, 15

minutes, at 4°C. The CAT activity in the cell extract was determined by a two-phase fluor diffusion assay (Nachtigal et al., 1989). Also, the DNA was isolated from the insoluble material and 2 μg samples were placed in duplicate on nitrocellulose membrane via slot-blot apparatus and probed with ^{32}P-labelled CAT insert DNA. Autoradiograms were analyzed by densitometry and all CAT activity data was normalized for transfection efficiency. For transgenic mice, a total cellular extract of each tissue was assayed for CAT activity.

Characterization of the Mechanism of Androgen Action

The PB DNA sequence comprising nucleotides -426 to +28 (Figure 1) contains the necessary information to obtain androgen regulation, as defined below. The PB-CAT constructs, deletions, and mutations therein, were used to determine the functional significance of ARE-1 and ARE-2.

Due to the lack of appropriate cell lines containing either androgen receptors and/or prostatic in origin, a range of cell lines was tested. The human prostatic cells LNCaP would express and androgen regulate PB-CAT- while HeLa, prostatic DU-145 and PC-3 cells required co-transfection of an androgen receptor expression vector for androgen regulation. The PC-3 human prostatic carcinoma line transiently transfected with the hybrid PB-CAT, co-transfected with the rat androgen receptor expression vector was the standard assay using dihydrotestosterone (DHT) or R1881 (a synthetic androgen). Dexamethasone (DEX), a synthetic glucocorticoid was studied by cotransfecting PC-3 cells with an expression vector for glucocorticoid receptor (GR).

A series of 5'-deletions of PB-CAT DNA were constructed and transfected into PC-3 cells. Deletion of the PB 5'-flanking DNA between -426 and -286 resulted in a net increase in androgen inducible CAT activity (Figure

2). A similar observation was made with cells transfected with GR expression vector and treated with DEX (Figure 2). The increased activity up to deletion -286 implies that there was a *cis*-acting silencer upstream. At -286 PB-CAT, the absolute net level of androgen-induced CAT activity (4605 dpm/min/mg protein) as well as a 42-fold increase from basal was higher than the net activity (496 dpm/min/mg protein) and 29-fold induction seen with DEX. At a further deletion to position -244, steroid induction of CAT activity was reduced. Progesterone treatment with cotransfection of the chicken progesterone expression vectors show little activity on -286PB-CAT. The PB AREs preferentially respond to androgens as compared to glucocorticoids and poorly to progestins in both PC-3 and HeLa cells (Table I) which indicates that the PB AREs function as a distinct androgen responsive element rather that as a GRE which is permissive for androgen receptor.

Further analysis of the 5′ and 3′ PB flanking regions demonstrates that the two *cis*-acting DNA elements which bind the androgen receptor, ARE-1 and ARE-2 are both required for androgen induced CAT activity (Rennie et al., 1993). This was demonstrated by making a mutation in ARE-1, at base -231 (G) being changed to A (Mut 1) or within ARE-2, at base -123 (C) being changed to an A (Mut 2) (Figure 3). Our data also indicates that preferential androgen action requires not only a specific/tight AR binding to the two sites but also interactions with adjacent DNA sequences and proteins. This further mediates cooperativity between AR binding and leads to preferential induction by AR. The two ARE sites function in a cooperative manner for the binding of AR (Table II). AR binds at high affinity when both ARE-1 and ARE-2 are present with the endogenous PB promoter (-286 to +28 bp) as demonstrated by the footprint which occurs at 60 ng of synthetic GST-AR2. Removal of either

ARE-1 (-157 to +28 bp) or ARE-2 (-426 to -134 bp) reduces binding of GST-AR2 to the remaining site (Table II). Single base mutations in ARE-1 (Mut 1) or ARE-2 (Mut-2) reduce steroid induced CAT bioactivity by >95% (Figure 3) and reduce binding of the GST-AR2 to both AREs (Table II). These two sites require flanking DNA sequences to confer preferential androgen regulation since removal of the endogenous PB promoter (replacing it with thymidine kinase promoter, TK) results in constructs that bind AR only at higher concentrations (Table II) and now are equally inducible by the synthetic glucocorticoid, DEX (Table III). Also, by removing the DNA sequence between ARE-1 and ARE-2 (construct contains ARE-1 placed adjacent to PB position -158 bp which contains the endogenous PB promoter), we see that DEX becomes a potent stimulus to induce CAT activity 21-fold which is similar to DHT induction of 16-fold (Table III).

We have demonstrated the utility of the AREs in the -286PB-CAT gene as a bioassay for defective androgen receptor seen in Androgen Insensitivity Syndrome (Figure 4) (Kazemi-Esfarjani et al., 1993). Differences between the wild type AR and AIS ARs are best recorded a lower concentrations of androgens (1 nM or less) while at high concentrations (10 nM), the wild type AR and V865L show similar response. Our assay also will measure the potency of an anti-androgen material when it is added to compete any androgenic activity. To increase the sensitivity and specificity of the bioassay, two additional improvements have been made.

1) -286 PB-ARE2*CAT: The sequence within ARE-2 was changed by substitution of four bases at -130 to -127 from CCAA with -130 to -127 TACT or GTCT resulting in ARE2* (see Figure 1). These constructs shows an increased response to androgens such that the change gave 100-fold induction compared to wild type -286PB-CAT which gave 36-fold induction. Further, this ARE2* showed an

increased response for DEX of 19-fold compared to wild type -286PB-CAT which gave 9.6-fold. The -286 PB-ARE2*CAT also demonstrated that androgen specificity can be separated further from the glucocorticoid inducible effect resulting in enhanced bioactivity and increased sensitivity. For example, androgens plus androgen receptor or plus defective androgen receptor as found in AIS show a greater difference with ARE2* constructs when compared to the wild type -286 PB-CAT (Table IV).

2) (-244/-96)nPB-TK. Although DHT and DEX function equally well and androgen specificity is lost with the replacement of PB promoter region (-96/+28) by TK (either -109 bp or -81 bp TK), the gain in the bioassay has been that the TK promoter functions in all cells tested. In order to regain androgen specificity while keeping the TK promoter, the -244/-96 has been arranged 5′ to 3′ as repeats adjacent to -109 or -81 TK where both TK promoters function equally well for induction by steroids but the -81 TK starts with a very low basal activity. The CAT gene when replaced by the Luciferase (Luc) reporter increases the sensitivity of the assay (De Wet et al., 1987). The constructs are (-244/-96)nPB-81TKLuc where n equal the number of repeats. When n=2 or n=3, a potent androgen response is obtained with only a small increase as n increases further when 5 μg of DNA construct is transfected (Table V). These constructs show increased sensitivity to measuring the activity of the androgen receptor, of androgens at very low concentrations (Table VI), and of defects in the AR as seen in AIS (Table VII).

Routinely, three repeats (-244/-96 PB) give a >300-fold induction in response to androgens (Table VI and VII) and show specificity for androgens over DEX at very low concentrations of steroids while at high concentrations of steroids, both DHT and DEX show equal activity (Table VI). This indicates the assay is not

limited to androgens but also can measure other steroid activities at higher concentrations of these steroids (>1 nM). A combination of either four base change described for ARE2* in each repeat of -244/-96 PB will further increase the sensitivity to steroids.

2) Transgenic non-human eukaryotic animal models for prostatic disease

In order to establish new animals models for prostatic carcinogenesis and benign prostatic hyperplasia, the PB sequence (Figure 1) can be used to target any gene to the prostate. We have proven that the PB 5'-flanking region contains the necessary sequences for prostatic targeting in transgenic mice by demonstrating that PB will direct prostate specific expression of the bacterial CAT gene (Greenberg et al., 1992a; Greenberg et al., 1992b; Greenberg et al., 1993; Greenberg et al., 1993). Prostate specific expression and androgen regulation in transgenic animals of the PB targeted gene is described.

In vivo studies have demonstrated that the PB promoter targets prostate-specific expression in transgenic mice. Using the -426/+28 PB-CAT construct as the transgene, 5 of 21 pups born following microinjection were identified by PCR as carrying the PB-CAT transgene (4 males, 1 female). Lines were established with the founder transgenic mice and were shown to transmit the same pattern of PB-CAT expression to the prostate of subsequent generations. Three of the male transgenic lines showed prostate-specific CAT expression while no CAT activity was detected in any tissue of one male and the female. Transgenic line #4248 (Table VIII) shows a high level of CAT activity in the prostate while line #4217 shows the lowest level but the same prostate-specific expression of CAT activity. The third male had intermediate CAT levels. The variability in the level of transgenic CAT expression among lines, including the lack

of expression in one male founder, may be due to the site of transgene integration. Further characterization of the highly expression transgenic line (#4248) demonstrates extremely high CAT activity in the lateral lobe (Using only 5 ug of tissue extract results in 21% of the substrate being acetylated, while at the usual 25 ug of extract, 89% of the substrate is acetylated). The fact that the PB gene's promoter functions so well is consistent with the high level of expression of the endogenous PB gene in the lateral prostate (8% of the total mRNA). The male accessory organ distribution of CAT activity in the transgenic mouse (true of all three lines, line #4248 in Table IX) closely parallels the endogenous rat PB mRNA levels reported (Matusik et al., 1986). Further, the *in situ* hybridization and immunohistochemistry of CAT reveal that the PBCAT gene is expressed in the same epithelial cells that was reported for PB mRNA and protein (Spence et al., 1989; Sweetland et al., 1988).

Further, the PBCAT gene demonstrates developmental and androgen regulation of expression. By 7 weeks of age in 3 males of 5 founders mice (4 males, 1 female), the CAT gene was preferentially expressed in the lateral, dorsal, and ventral prostate. Again as the animals age (to 23 week old), only low levels were detected in the anterior prostate and seminal vesicles and no CAT activity was detected in the brain, kidney, spleen, lung, heart, thymus, liver, or testis of any line. However, as the animals aged in line #4248, expression of PB-CAT increased in the ventral prostate up till the last time point checked (23 weeks), decreased in the lateral lobe, showing little change in the dorsal lobe. The CAT activity in the prostate ranged over several logs between the lowest and highest expressing mouse lines, likely due to the site of transgene integration. This expression pattern has been passed over 4 generations. The high

expressing male founded line #4248 showed a 70-fold increased prostatic CAT activity between 3 to 7 weeks of age, a time corresponding to sexual maturation. By 7 days after androgen removal (castration of mature males, line #4248), prostatic CAT activity declined (Table X) and could be induced by androgen replacement but not DEX (Table X). In subsequent studies, PB-CAT was coinjected with chicken lysozyme gene matric attachment region, MAR, (McKnight et al., 1992) and cointegration of the MAR and PB-CAT resulted in dorsolateral prostate-specific CAT expression in all three lines examined. With the addition of MAR, no ventral expression was detected in transgenic mice.

This demonstrates the specificity and androgen regulation of the PB 5'-flanking sequences with the CAT reporter transgene in transgenic non-human eukaryotic animals. To enhance the androgen regulation, the ARE2*, repeats of -244/-96 PB, and/or ARE2* placed into repeats of -244/-96 can be added to the -426/+28 PB promoter. To make the promoter responsive to metal ions, such as Zn and Cd, a MT inducible elements, such as used in transgenic animals (Dyer et al., 1989; Iwamoto et al., 1992; Li et al., 1989; Russo et al., 1988; Mayo et al., 1988; Iwamoto et al., 1991) or to make it responsive to glucocorticoids, the GRE sequences, such as seen in the MMTV promoter (Stamp et al., 1992; Lin et al., 1992; Lucchini et al., 1992; Bouchard et al., 1989; Muller et al., 1988; Leder et al., 1986), can be added. The PB 5'-flanking sequence can be used to target any gene to the prostate that may change prostatic function, growth, or cause tumor formation. The targeted genes include large T, TRPM-2, bcl-2, mutated p53, myc, ras, bFGF, TGF-β1, activin, activin receptor, AR, RXR, c-fos, IGFs, IGFBPs, PSA, and int-2. Further, transgenic lines bearing different PB-targets genes can be crossed to develop new lines that show a different incidence or type of tumor

development. In this manner, genes important for prostatic tumor growth can be identified. In addition, PB-targeted genes may work in combination with other endogenous genes or newly activated genes to induce tumor growth. The PB transgenic model permits identification of these genes.

3) Cell culture models for prostatic disease.

The transgenic mouse models developed will enable researchers to study and dissect the multistep process of tumorigenesis as it occurs *in vivo*. These investigations will yield relevant histological and pathological correlates with the known transgenic phenotype in the context of the whole animal. However, whole animal studies are not always adaptable to delineate the various interactions materials, such as hormones, growth factors, attachment factors and cytokines, which affect growth rate, differentiation, metastatic potential and phenotypic expression. In order to address these parameters, a rapid *in vitro* model for assays can be established. Transgenic animal lines, produced with the PB targets genes which then show prostatic overgrowth or tumor formation, are used as a source of tissue to isolate cells for the establishment of replicative cell cultures (cell lines). Immortalized cells taken from transgenic animals have been successfully used to establish cell culture lines (Larue et al., 1993; Hammang et al., 1990; Martinez de la Escalera et al., 1992; Mellon et al., 1992; von Deimling et al., 1990; Windle et al., 1990; Dalemans et al., 1990; Galiana et al., 1990; Vaux et al., 1988; Efrat et al., 1988; Anonymous, 1991; Tal et al., 1992).

4) Treatment of human benign prostatic hyperplasia and human prostate cancer

The treatment of human disease by gene therapy can be applied to human benign prostatic hyperplasia (hBPH), prostate cancer (CaP), and any disease state of the

prostate by using the ability of probasin to target a toxin to prostatic cells. Our studies described in this patent demonstrate that PB directs expression to the transgenic animal prostate and PB directs expression in human prostatic cancer cell lines. Since transgenes coupled to the probasin promoter would be targeted for expression specifically to the prostatic cells, side effect of the therapy on other cell types would be limited. In CaP, stable integration of the PB-targeted transgene into patient chromosomal DNA would not be required since the goal is to kill the cancer cells. Therapy for hBPH may be designed to kill the hyperplastic cells or to integrate the PB-targeted transgene to correct or reduce the hyperplastic growth.

SUMMARY OF DISCLOSURE

In summary of this disclosure, the present invention provides a novel DNA sequence which permits establishment of an assay for androgenic and anti-androgenic materials, transgenic animals and gene therapy for treatment of the prostate.

Features of the present invention described herein and provides hereby include the following:

1. By transient transfected or stably integrated PB sequences coupled to a reporter gene, a bioassay for androgenic materials can performed in cell culture. The androgenic material is not limited to ligands for the androgen receptor but may include non-steroid pathways that result in androgen action, proteins, DNA and RNA sequences important for androgen action.

2. The transfected cells of feature 1 can be used to bioassay any anti-androgenic material by competition studies with androgenic material. The anti-androgenic material includes receptor ligands, proteins, DNA and RNA sequences, materials the may alter the phosphorylation state, and alter the pathway of androgen action. The anti-androgen material may interfere in the androgen

action pathway by direct binding of the ligand to the steroid receptor, it may bind directly to the steroid receptor or PB DNA sequences thus interfering with receptor binding to DNA, RNA, and/or proteins, or it may affect modification of material in the pathway of androgen action.

3. Androgenic and anti-androgenic materials can be assayed by determining their effect on the binding of androgen receptor to PB DNA sequences or to protein complexes bound with the androgen receptor to PB DNA sequences.

4. In transgenic non-human eukaryotic animals, the PB sequences target genes to the male urogenital tract with the highest level of expression in prostatic cells. Gene(s) would be integrated into the chromosome of the animal by introduction at the embryonic stage.

5. In non-human eukaryotic animals, the PB sequences can target genes to the male urogenital tract after injecting DNA via viral or non-viral methods resulting in the highest level of expression in prostatic cells.

6. In animals of feature 4 or 5, the PB promoter regulates expression of gene(s). Transcription via the PB promoter may be further regulated by the addition of enhancer, inducible, or repressor DNA elements.

7. In animals of feature 4 or 5, genes targeted can induce prostatic disease including prostatitis, hyperplasia, urethral obstruction, and prostate cancer to serve as models.

8. From animals of feature 7, cells can be isolated from prostatic tissue to establish cell lines.

9. In animals of feature 4 or 5 or cell lines of feature 8, therapies may be tested to develop new drugs including new approached to gene therapy.

10. The predisposition of animals of features 4 or 5 or cell lines of feature 8 for prostatic disease can be

used to test materials that have protective value against the development or progression of prostatic disease. A lower incidence of prostatic tumor development would demonstrate a protective value of an agent.

11. The predisposition of animals of feature 4 or 5 or cell lines of feature 8 to prostatic disease increases the sensitivity to measure materials for carcinogenicity. Transgenic animals lines or cell culture lines can be selected that have a low susceptibility of developing prostatic tumors and treated with a potential carcinogen over a range of doses. Further, lines which develop tumors rapidly would increase the sensitivity of the test to weak carcinogens. A carcinogen should show an increase in tumor development and or progression over controls.

12. In animals (transgenic as well as non-transgenic), the PB sequences can be used as a model to develop methods for new gene therapies by the delivery of genes which express toxins or convert drugs into toxic substances in normal prostatic, hyperplastic, and cancerous cells.

13. In humans, the PB sequences can be used in gene therapy to target genes to the male urogenital tract, benign prostatic hyperplasia, and prostatic cancer. Targeted genes can express toxins or converted drugs into toxic substances thereby inhibiting the growth or killing prostatic cells.

Modifications are possible within the scope of this invention.

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TABLE I

Hormonal Induction of PB-CAT				
Cell Type Used	Type of Steroid Receptor Cotransfected with PB-CAT	CAT Activity (dpm/min/mg protein)		
		-Steroid	+Steroid	Net
HeLa	Androgen	1.5 ± 0.8	17 ± 5	15.5
	Glucocorticoid	0.9 ± 0.4	17 ± 3	17.1
	Progestin*	95 ± 26	68 ± 6	0.0
PC-3	Androgen	105 ± 21	884 ± 105	779
	Glucocorticoid	17 ± 6	72 ± 8	55
	Progestin*	29 ± 10	115 ± 9	86
CAT activity is expressed as mean ± SEM for n>3 *Transfection with -286 PB-CAT rather than -426 PB-CAT				

TABLE II

Appearance of Footprint with AR (ng) on the Probasin Promoter		
PB Fragment (bp)	ARE-1	ARE-2
-286 to +28	60 ng	60 ng
-426 to -134	200 ng	ARE-2 absent
-157 to +28	ARE-1 absent	100 ng
-244 to -96	200 ng	200 ng
Mut 1 (-286 to +28)	suggestive*	200 ng
Mut 2 (-286 to +28)	suggestive*	200 ng

Mut 1 contains a mutation in ARE-1 that deceases DHT induced CAT bioactivity by >95%

Mut 2 contains a mutation in ARE-2 that deceases DHT induced CAT bioactivity by >95%

* Suggestive indicates that a weak footprint occurs at >400 ng GST-AR2 but always lacks the complete "white out" appearance seen in all other AR footprints.

TABLE III

Bioactivity and Specificity for AR Depends upon both AREs and adjacent DNA Sequences in the PB Fragment						
CAT Construct with either PB* or TK promoter	CAT Activity (dpm/min/mg protein)					
	-DHT	+DHT	Fold	-DEX	+DEX	Fold
-244/+28 PB-CAT	5.9	288	49	6.2	55.5	9
-158/+28 PB-CAT	6.3	8.5	1.4	6.0	7.7	1.2
(ARE-1)-158/+28 PB-CAT	6.1	108	18	6.2	131	21
-244/-265 PB TKCAT	4.8	5.4	1.1	7.4	10.2	1.3
-244/-96 PB TKCAT	6.7	410	61	7.0	672	96

*PB denotes the fragment of probasin flanking DNA.
(ARE-1) refers to the precise sequence placed adjacent to -158 PB-CAT.
Fold refers to the increase when steroid is added.

TABLE IV

A Four Base Pair Change in ARE-2 Increases the Sensitivity for Altered Androgen Receptor Seen in AIS		
DNA Construct	-286 PB-CAT	-286 PB-ARE2*CAT
wild type hAR	50^	177^
V865L hAR	49^	122^
V865M hAR	42^	49^
^CAT activity expressed as dpm/min/mg protein. Background CAT activity without androgens is 4.2. Bioassay shown is induced levels with 10 nM DHT.		

TABLE V

Repeats of a PB Fragment Containing both ARE-1 and ARE-2			
(-244 to -96)n PB adjacent -81 TK-Luc*	Luc Activity Relative Light Units (RLU) x 10^6 per min/mg protein		
	-DHT	+DHT 10nM	Fold
n=1	0.07	1.31	18
n=2	0.09	14.5	161
n=3	0.09	27.7	308
n=4	0.08	23.7	296
n=5	0.09	26.2	290

*The -81 TK-Luc vector when tested as a control showed only a 1.8 increase with the addition of DHT. All repeats of PB are placed adjacent to each other in the same 5'-3' direction as wild type probasin.

TABLE VI

Repeats of the PB AREs Increase Sensitivity to Low Levels of Androgens				
Agent Tested		(-244 to -96)n PB -81 TK-Luc		
Steroid*	Concentration	n=1	n=2	n=3
R1881	1 nM	11	230	245
DEX	1 nM	3	39	130
R1881	0.1 nM	9	172	251
DEX	0.1 nM	1.1	6	18
R1881	0.01 nM	2	15	24
DEX	0.01 nM	0.8	1.1	1.1

*The steroids are R1881 a synthetic androgen and DEX a synthetic glucocorticoid. Data is expressed as fold change from baseline when steroid is added.

TABLE VII

Repeats of the PB AREs Increase Sensitivity to Defects in the AR as seen in AIS			
DNA	(-244 to -96)n PB -81 TK-Luc		
Construct	n=1	n=2	n=3
wild type hAR	13.6	256	832
V865L hAR	11.6	146	375
V865M hAR	6	54	103
Data is presented as fold increase of Luc activity from baseline with the addition of 10 nM DHT.			

TABLE VIII

Expressed CAT Activity in PB-CAT Trangenic Mouse Line #4248	
TISSUE	CAT ACTIVITY*
Brain	0.08
Kidney	0.07
Spleen	0.09
Lung	0.07
Heart	0.09
Thymus	0.15
Liver	0.12
Testis	0.13
Seminal Vesicle	1.01
Lateral Prostate	37.69
Dorsal Prostate	2.32
Ventral Prostate	8.35
Anterior Prostate	0.23
* CAT activity is expressed as conversion of substrate in pmol/hr/mg protein.	

TABLE IX

Relative Concentration (Per Cent) of Expressed mRNAs in Various Tissues		
TISSUE	ENDOGENOUS RAT PROBASIN	TRANSGENIC MOUSE PB-CAT
Lateral Prostate	100	100
Dorsal Prostate	33	6
Ventral Prostate	4	22
Anterior Prostate	14	0.6
Seminal Vesicle	2	2.7
Testis	ND*	ND
Brain	ND	ND
Heart	ND	ND
Liver	ND	ND
*ND=Not Detected		

TABLE X

Hormonal Regulation of PB-CAT Expression in Transgenic Mice: Line 4248			
TREATMENT	LATERAL PROSTATE	DORSAL PROSTATE	VENTRAL PROSTATE
Intact: 8 weeks old	10,212 ± 4,897	2,539 ± 812	7,905 ± 1,971
Intact: 9 weeks old	12,083 ± 3,168	2,346 ± 630	11,787 ± 1,782
Castrated for 7 Days	529 ± 600	374 ± 91	218 ± 173
Castrated for 10 Days	1,364 ± 606	224 ± 261	2,061 ± 533
Castrated for 7 Days + Test for 3 Days	8,289 ± 1,855	1,222 ± 569	7,553 ± 4,075
Castrated for 7 Days + DEX for 3 Days	1,646 ± 832	187 ± 66	1,435 ± 228
CAT activity is expressed as dpm/min/mg protein ± standard error. Test = testosterone; DEX = dexamethasone			

SEQUENCE LISTING

INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 556 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 467..547

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
AAGCTTCCAC AAGTGCATTT AGCCTCTCCA GTATTGCTGA TGAATCCACA GTTCAGGTTC      60

AATGGCGTTC AAAACTTGAT CAAAAATGAC CAGACTTTAT ATTCTTACAC CAACATCTAT     120

CTGATTGGAG GAATGGATAA TAGTCATCAT GTTTAAACAT CTACCATTCC AGTTAAGAAA     180

ATATGATAGC ATCTTGTTCT TAGTCTTTTT CTTAATAGGG ACATAAAGCC CACAAATAAA     240

AATATGCCTG AAGAATGGGA CAGGCATTGG GCATTGTCCA TGCCTAGTAA AGTACTCCAA     300

GAACCTATTT GTATACTAGA TGACACAATG TCAATGTCTG TGTACAACTG CCAACTGGGA     360

TGCAAGACAC TGCCCATGCC AATCATCCTG AAAAGCAGCT ATAAAAAGCA GGAAGCTACT     420

CTGCACCTTG TCAGTGAGGT CCAGATACCT ACAGAGCTCA CACAGC ATG AGG GTC        475
                                                    Met Arg Val
                                                      1

ATC CTC CTC CTG CTC ACA CTG GAT GTG CTA GGT GTC TCC AGT ATG ATG        523
Ile Leu Leu Leu Leu Thr Leu Asp Val Leu Gly Val Ser Ser Met Met
  5                  10                  15

ACA GAC AAG AAT CTC AAA AAG AAG GTAGCAGAC                             556
Thr Asp Lys Asn Leu Lys Lys Lys
 20                  25
```

INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Met Arg Val Ile Leu Leu Leu Leu Thr Leu Asp Val Leu Gly Val Ser
 1               5                  10                  15

Ser Met Met Thr Asp Lys Asn Leu Lys Lys Lys
            20                  25
```

INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TACT 4

INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GTCT 4

INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CAAT 4

INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TATAA 5

INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

ATAGCATCTT GTTCTTAGT 19

INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GTAAAGTACT CCAAGAACCT ATTT 24

CLAIMS

1. An isolated DNA molecule comprising a 5'-flanking region of the rat probasin gene and containing at least one androgen responsive element.

2. The DNA molecule of claim 1 comprising two androgen responsive elements.

3. The DNA molecule of claim 2 wherein both said androgen responsive elements contain the conserved sequence 5'-GTTCT.

4. The DNA molecule of claim 1 wherein said at least one androgen responsive element has the sequence ATAGCATCTTGTTCTTAGT (ARE1).

5. The DNA molecule of claim 1 wherein said at least one androgen responsive element has the sequence GTAAAGTACTCCAAGAACCTATTT (ARE2).

6. The DNA molecule of claim 1 having the sequence shown in Figure 1 or one which hybridized thereto under stringent conditions.

7. The DNA molecule of claim 1 wherein the probasin promoter region (-95 to +28) is replaced by a TK promoter.

8. The DNA molecule of claim 1 comprising a repeated (-244 to -96) region of the 5'-flanking region linked to the TK promoter.

9. An isolated DNA molecule comprising an androgen responsive element of the rat probasin gene or a mutation thereof having retained or modified androgen activity.

10. The DNA molecule of claim 9 wherein said DNA molecule comprises nucleotides -241 to -223 (ARE1) as seen in Figure 1 or a mutation thereof having reduced androgen activity.

11. The DNA molecule of claim 9 wherein said DNA molecule comprises nucleotides -140 to -117 (ARE2) as seen in Figure 1 or a mutation thereof having reduced androgen activity.

12. The DNA molecule of claim 9 wherein said DNA molecule comprises nucleotides -140 to -117 (ARE2) as seen in Figure 1 in which nucleotides -130 to -127 are replaced by nucleotides TACT or GTCT having increased androgen activity.

13. A transgenic non-human eukaryotic animal having a genomic integration of a DNA molecule comprising a 5'-flanking region of the rat probasin gene and containing at least one androgen responsive element permitting prostate-specific heterologous gene expression under androgen regulation.

14. The transgenic animal of claim 13 wherein transcription via the probasin promoter is further regulated by the presence of enhancer, inducible and/or repressor DNA elements.

15. The transgenic animal of claim 14 wherein said DNA elements comprise genomic integration of MT- and/or GRE-inducible elements linked to probasin sequences.

16. A non-human eukaryotic animal having administered thereto, by viral or non-viral methods, a DNA molecule comprising the 5'-flanking region of the rat probasin gene permitting heterologous gene expression and containing at least one androgen responsive element and resulting in expression in prostatic cells or cells prostate in origin in the animal.

17. A cell line comprising cultured prostatic tissue from a non-human eukaryotic animal as claimed in claim 13 or 16.

18. A non-human eukaryotic animal or a cell line derived from prostatic tissue of said eukaryotic animal predisposed to prostatic disease by a DNA molecule comprising the 5'-flanking region of the rat probasin gene and containing at least one androgen responsive element.

19. A method of treatment of a human disease state of the prostate by gene therapy, which comprises targeting

of a gene to prostate cells by employing a DNA molecule containing the 5'-flanking region of the rat probasin gene.

20. The method of claim 19 wherein said disease state is human benign hyperplasia or prostate cancer and said gene codes for a substance for treatment of said disease state.

21. A bioassay for androgenic and anti-androgenic materials, which regulates a DNA molecule comprising a 5'-flanking region of the rat probasin gene and containing at least one androgen responsive element coupled to a reporter gene.

22. The bioassay of claim 21 wherein said materials is tested on transiently transfection of said DNA molecule.

23. The bioassay of claim 21 wherein said materials is tested with said DNA molecule stably integrated into cells.

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AAGCTTCCACAAGTGCATTTAGCCTCTCCAGTATTGCTGATGAATCCACAGTTCAGGTTC

AATGGCGTTCAAAACTTGATCAAAAATGACCAGACTTTATATTCTTACACCAACATCTAT

CTGATTGGAGGAATGGATAATAGTCATCATGTTTAAACATCTACCATTCCAGTTAAGAAA

ATATGATAGCATCTTGTTCTTAGTCTTTTTCTTAATAGGGACATAAAGCCCACAAATAAA

AATATGCCTGAAGAATGGGACAGGCATTGGGCATTGTCCATGCCTAGTAAAGTACTCCAA

-101 -95 -82 -75

GAACCTATTTGTATACTAGATGACACAATGTCAATGTCTGTGTACAACTGCCAACTGGGA

-48 -27

TGCAAGACACTGCCCATGCCAATCATCCTGAAAAGCAGCTATAAAAAGCAGGAAGCTACT

+1 +28

CTGCACCTTGTCAGTGAGGTCCAGATACCTACAGAGCTCACACACG ATG AGG GTC
Met Arg Val

ATC CTC CTC CTG CTC ACA CTG GAT GTG CTA GGT GTC TCC AGT
Ile Leu Leu Leu Leu Thr Leu Asp Val Leu Gly Val Ser Ser

ATG ATG ACA GAC AAG AAT CTC AAA AAG AAG GTAGCAGAC
Met Met Thr Asp Lys Asn Leu Lys Lys Lys

FIGURE 1

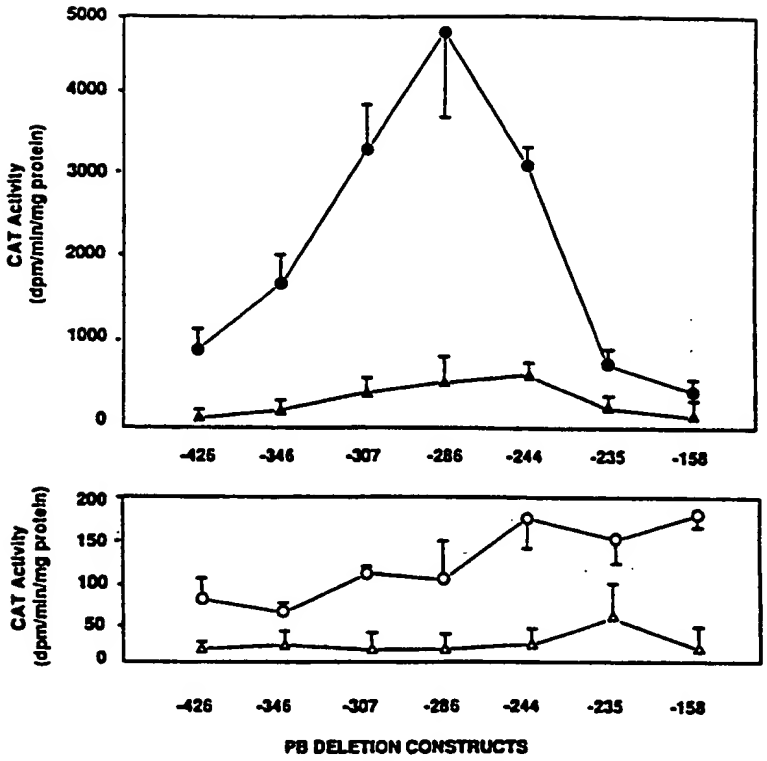
5000
4000
3000
2000
1000
0
CAT Activity
(dpm/min/mg protein)
-425
-346
-307
-286
-244
-235
-158
200
150
100
50
0
CAT Activity
(dpm/min/mg protein)
-426
-346
-307
-286
-244
-235
-158
PB DELETION CONSTRUCTS

FIGURE 2

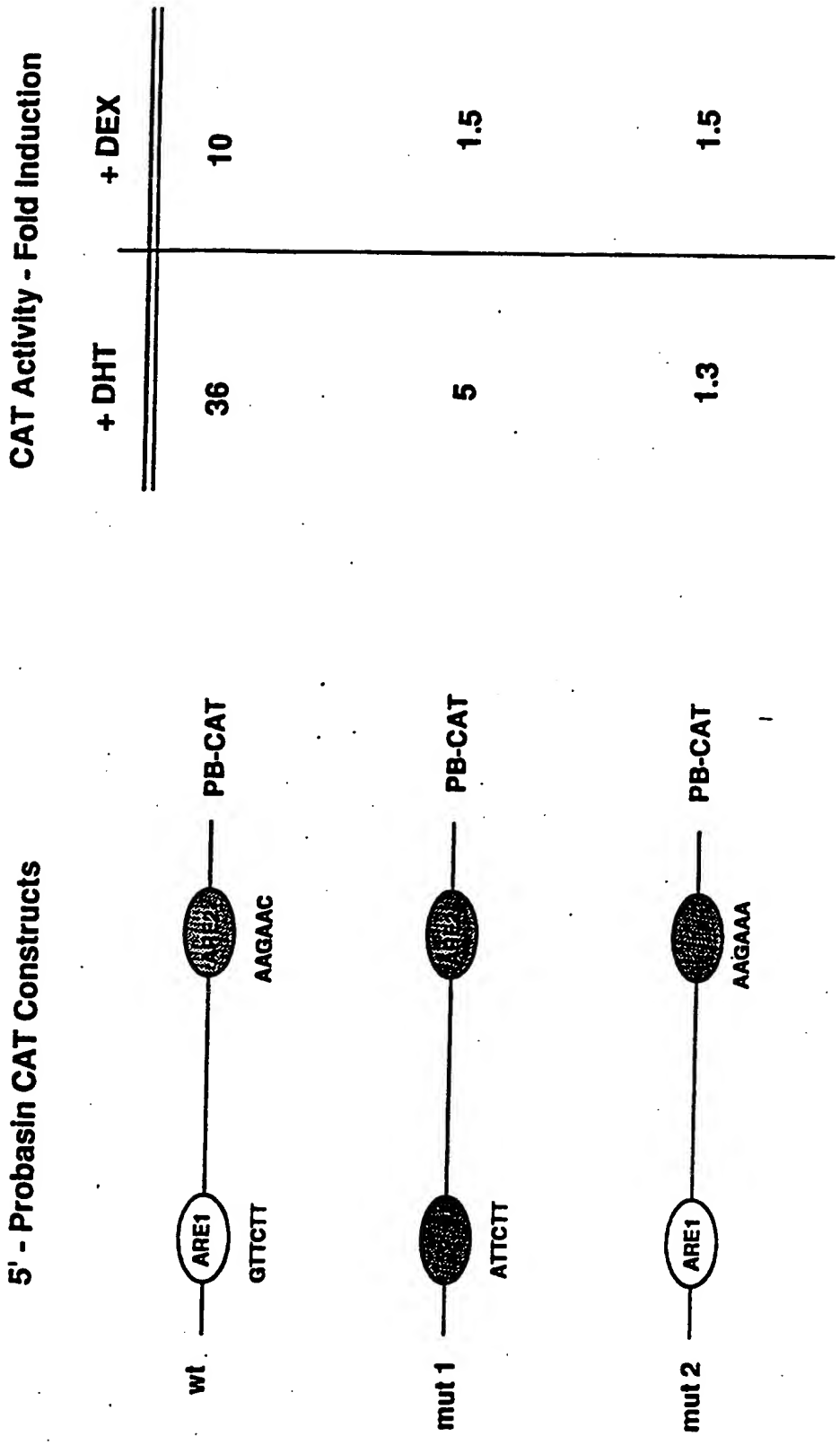
CAT Activity - Fold Induction
+ DHT
+ DEX
36
10
5
1.5
1.3
1.5
5' - Probasin CAT Constructs
wt
ARE1
GTTCTT
AAGAAC
PB-CAT
mut 1
ATTCTT
PB-CAT
mut 2
AAGAAA
PB-CAT

FIGURE 3

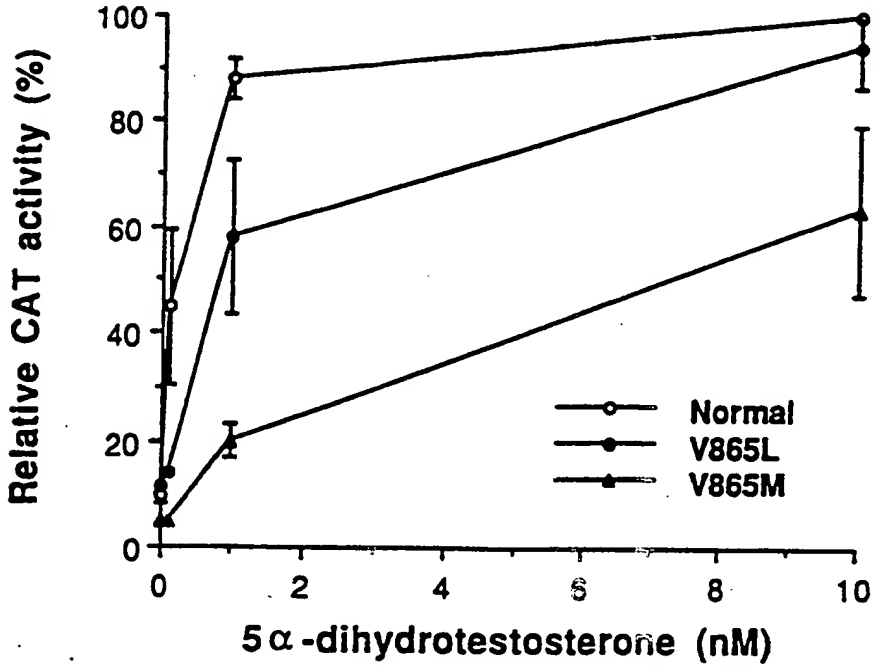
Relative CAT activity (%)
100
80
60
40
20
0
0
2
4
6
8
10
5α-dihydrotestosterone (nM)
Normal
V865L
V865M

FIGURE 4

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/00 A01K67/027 C12N15/12 A61K48/00 C12N15/85
G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 A01K C07K A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 86, no. 20 , 1989 , WASHINGTON US pages 7843 - 7847 SPENCE, A.M. ET AL. 'Regulation of a bifunctional messenger RNA results in synthesis of secreted and nuclear probasin' see the whole document ---	1
A	IN VITRO CELL DEVELOPMENT BIOLOGY vol. 25, no. 6 , 1989 pages 581 - 584 MATUO, V. ET AL. 'The androgen-dependent rat prostate protein is a heparin-binding protein that co-purifies with heparin-binding growth factor-1' see the whole document --- -/--	1

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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Date of the actual completion of the international search
10 November 1993

Date of mailing of the international search report
22 -12- 1993

Name and mailing address of the ISA
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CHAMBONNET, F

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,90 09443 (HARVARD COLLEGE) 23 August 1990 see claims	1

2

INTERNATIONAL SEARCH REPORT

tional application No.

PCT/CA93/00319

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 19 and 20 are directed to a method of treatment of the human body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos..
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9009443	23-08-90	US-A-	5175383	29-12-92
		AU-A-	5278590	05-09-90
		EP-A-	0458908	04-12-91
		JP-T-	4505704	08-10-92